SPECIFICATION

PREVENTIVE AND THERAPEUTIC COMPOSITIONS FOR DRUG-INDUCED NEPHROPATHY AND HEPATOPATHY

Technical Field

The present invention relates to drugs for relieving drug-induced nephropathy and hepatitis.

Background Art

The use of drugs for therapeutic and/or diagnostic purposes has increased year by year, and the drugs used have diversified. These drugs can provide us significant benefits but can also cause substantially harmful effects, especially to kidneys, due to their specific functions described below.

Kidneys weigh less than one percent of the total body weight. From a physiological viewpoint, 25 percent of the total cardiac output flows into the kidneys; 150 liters of primitive urine, up to 50 times the total blood plasma, is filtered through glomeruli per day; and final urine is made by reabsorption, secretion, and metabolism through uriniferous tubules variable in structural and functional heterogeneity. Thus, drugs or their metabolites in blood always circulate, and these substances are concentrated and metabolized in kidneys. Consequently, various highly concentrated metabolites, including original drugs, are distributed in kidneys. Kidneys are likely to be frequently and intensively exposed to drugs. Four types of drugs may induce nephropathy: antimicrobial agents, nonsteroidal agents, contrast agents, and antitumor agents.

The liver can also be easily damaged by drugs. Drug-induced hepatopathy is classified by its onset mechanism into toxic hepatopathy caused by direct attack of drugs or their intermediate metabolites to the liver, and allergic hepatopathy caused by allergic response, type IV delayed allergic response in which T cells are involved. Drug-induced hepatitis is caused by, most frequently, antibiotics, followed by drugs for the central nervous system, drugs for circulatory organs, antitumor agents, hormonal agents, diagnostic agents, etc.

Attempts have been made to relieve drug-induced disorders by using γ-globulin, cytochrome C, adenine, SH compounds, vitamin B group, etc., but they are not sufficiently effective. It is very important to clinically cure drug-induced disorders (side effects) because of the interruption of the treatment and the importance of patients' quality of life (Q.O.L.).

Disclosure of the Invention

An objective of the present invention is to drugs that effectively relieve or suppress disorders induced by various drugs, especially by antitumor agents.

The inventors have focused on the facts that proteins belonging to the midkine (MK) family such as midkine (MK) and pleiotrophin (PTN) are growth and differentiation factors with multiple functions. The functions include 1) elongation of neurite, 2) activation of fibrinolytic system, 3) strong expression in human cancerous areas, and 4) cure of wounds. Numerous studies have been performed on such proteins in order to find novel pharmaceutical effects.

Midkine was discovered as a product of the gene whose expression was induced in the early stage of the differentiation process with retinoic acid in mouse embryonic tumor cells (Kadomatsu, K. et al., Biochem. Biophys. Res. Commun., 151: 1312-1318, 1988). Pleiotrophin was discovered in the brain of a newborn rat as a heparin-binding protein with neurite elongation ability (Rauvala, H., EMBO J., 8: 2933-2941, 1989). Midkine and Pleiotrophin form a novel class of growth and differentiation factors as heparin-binding proteins. They exhibit 45% homology and are collectively called the MK family (Muramatsu, T., Int. J. Dev. Biol., 37: 183-188, 1993; Muramatsu, T., Develop. Growth & Differ. 36(1): 1-8, 1994). Midkine and Pleiotrophin each exhibits a specific expression pattern in development processes, and is expected to be involved in important physiological activation in differentiation.

The inventors found that MK inhibits cell death caused by antitumor agents *in vitro* and that MK gene relieves disorders induced by an antitumor agent from the results of an experiment in which an antitumor agent was administered to knockout mice in which MK gene

was functionally destroyed. The inventors also found that administering MK or PTN to wild mice relieves the disorders caused by antitumor agents, to complete the invention. The present invention encompasses each invention described in the claims.

In this invention, knockout mice provided an opportunity to investigate how MK gene in the living body fights against disorders caused by drugs and to analyze how each knockout mouse responds to the forced administration of MK at the individual level. Details of MK's function and mechanism are presently not clear. If MK functions as a trigger protein for the functional cascade of cytokines or growth factors, a very small amount of MK is presumably needed, and the use of knockout mice becomes more important. Recently, a cell surface receptor specifically binding to MK with high affinity (molecular weight 250 + kDa) has been discovered. Its characteristics imply that autocrine stimulated by MK in tumor cell proliferation could be mediated by the receptor and would activate the JAK/STAT pathway (Edward, A. R. et al., J. Biol. Chem. 273: 3654-3660, 1998).

To clarify the relationship between MK and ontogenesis, homozygous MK gene-knocked out mice in which parts of exon 2 and exon 3 are damaged as illustrated in Figure 1 were prepared (Biochemistry 7, Heisei 8: Volume 68, pp. 1239, 4-P-1244). Those knockout mice did not die during the fetal period and weighed significantly less than heterozygous or wild types (Biochemistry 7, Volume 68, pp. 1239, 4-P-1244, 1996).

Antitumor agents were administered to the knockout mice (simply referred as knockout mice) and wild mice. Survival rate, blood urea nitrogen (BUN) level, and creatinine level of each mouse were compared as indices of disorders after the administration to monitor the ability of MK gene in the living body to relieve disorders caused by antitumor agents. BUN and creatinine levels can be used as indices of functional disorders in kidneys because urea is accumulated in blood due to the reduced renal excretory ability.

In this invention, cisplatin was administered to the knockout mice and wild mice, then BUN level and survival rate were compared. The BUN level of the knockout mice were significantly higher than that of the wild mice. The survival rate of the knockout mice also

differed significantly from that of wild mice. The death rate of knockout mice increased by the seventh day after the administration. The rate of abnormal BUN levels in knockout mice that had been forcedly administered MK was significantly lower than that in the group that had been administered physiological saline by the third day after the administration. The effectiveness of MK in suppressing renal cell disorders caused by cisplatin was confirmed by conducting an experiment *in vitro* using human infantile renal cancer cell lines. An experiment using the wild mice revealed that MK relieved acute hepatopathy due to carbon tetrachloride, and that both PTN and MK effectively suppress nephropathy caused by cisplatin.

These results indicate that proteins of this invention belonging to the MK family effectively relieve or suppress drug-induced nephropathy and hepatopathy.

MK protein (simply referred to as MK) used as an effective ingredient of the pharmaceutical composition of the invention is described in the following references (human MK gene, unexamined published Japanese patent application (JP-A) No. Hei 5-91880; sequences of the human MK gene and protein, JP-A No. Hei 6-217778; MK protein, JP-A No. Hei 5-229957; Muramatsu, T., Develop. Growth & Differ. 36(1), 1-8, 1994). PTN protein used as an effective ingredient of the pharmaceutical composition of the invention is described in the following references (Muramatsu, T., Develop. Growth & Differ. 36(1), 1-8, 1994; Andreas, K. et al., Critical Reviews in Oncogenesis, 6(2): 151-177, 1995). The proteins belonging to the MK family and used as effective ingredients of the pharmaceutical composition of this invention include natural proteins derived from humans, mice, or other mammals, or artificial proteins manufactured by chemical synthesis or genetic engineering. Also, the proteins of the invention that belong to the MK family include proteins or polypeptides which do not cause any changes of the above-described biological activities and differ from proteins derived from nature in the number or the sequences of amino acids. Specifically, the present invention includes proteins corresponding to natural proteins in which the amino acid sequence of natural proteins is partially deleted or replaced by other amino acids, or other amino acids or

polypeptide of different length are inserted or added. Amino acids to be replaced or inserted are not limited to natural types.

The expression system using *E. coli* (Studier, F. W. & Moffatt, B. A., J. Mol. Biol. 189: 113-130, 1989; Studier, F. W. et al., Meth. Enzymol. 185: 60-89, 1990) or the expression system using baculovirus (O'Reilly, D. R. et al., Baculovirus Expression Vectors, A Laboratory Manual, Oxford University Press, 1992, Ausubel, F. M. et al. eds., Current Protocol in Molecular Biology, Unit 16.11, Wiley Interscience, 1994) can be used to obtain the MK family proteins that are effective ingredients of the invention using genetic engineering techniques. The inventors employed the expression system using methyl alcohol dependent yeast *Pichia pastoris* to obtain the MK proteins (refer to JP-A No. Hei 7-255354).

The pharmaceutical composition of the invention contains the MK family protein in an amount effective to prevent or treat nephropathy or hepatopathy caused by drugs. The effective ingredients of the invention can be prepared in a desirable dosage form by mixing with usually used pharmaceutically acceptable carriers, vehicles, diluents, preservatives, stabilizers, buffers, etc.

The pharmaceutical composition of the invention can be administered orally or parenterally. Dosage forms for oral administration include tablets, granules, and capsules. Dosage forms for parenteral administration include injection, suppositories, or percutaneous agents, which are administered intravenously, subcutaneously, intramuscularly, or intraperitoneally.

Physiologically active peptides such as MK or PTN are rapidly digested by protease in digestive tracts in general when they are administered orally. To stabilize MK or PTN *in vivo*, a hybrid MK or hybrid PTN should be prepared by binding it to water-soluble macromolecules (for example, polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP)). Hybrid constructions of IL-6, TNF-α, etc. have been attempted, and the function has been enhanced by selecting the most suitable hybrid condition (Tsutsumi, Y. et al., Br. J. Cancer. 74: 1090-1095; Tsutsumi, Y. et al., Thoromb. Haemostasis, 77: 168-173, 1997; Tsutsumi, Y. et al., J. Control Release, 33: 447-451, 1995).

The MK family proteins that are effective ingredients of the

pharmaceutical compositions of the invention vary depending on the dosage of the causative drug, severity of nephropathy or hepatopathy, age, sex, and weight of the patient when used to prevent or treat nephropathy or hepatopathy caused by drugs. The proteins of the invention can be administered once or several times at a dosage of 1 μg/kg to 100 mg/kg of body weight per day.

Brief Description of the Drawings

Figure 1 shows the mutant chromosome of the knockout mouse in which parts of exons 2 and 3 of MK gene of 129/Sv mice was destroyed.

Figure 2 shows the survival rate of 129/Sv MK knockout mice and that of wild mice after the administration of cisplatin.

Figure 3 shows blood urea nitrogen of 129/Sv MK knockout mice and that of wild mice on the day of administration and on the third and fifth days after the administration of cisplatin.

Figure 4 shows the frequency of abnormal blood urea nitrogen of 129/Sv MK knockout mice on the day of administration and on the third and fifth days after 14 mg/kg cisplatin was administered and MK or physiological saline absorbed capsules for sustained release were intraperitoneally implanted.

Figure 5 shows the serum GOT of mice suffering from acute hepatopathy caused by carbon tetrachloride when various concentrations of MK or physiological saline were administered to the mice.

Figure 6 shows the serum GTP of the same mice as in Figure 5.

Figure 7 shows the survival rate of G401 cells where 2×10^4 cells/well were cultured in the medium alone, the medium with 2 μg/ml of MK, or the medium with 10 μg/ml of MK, and treated with briplatin (cisplatin) during the culture.

Figure 8 shows the survival rate of G401 cells (6×10^4 cells/well) after the same treatment as in Figure 7.

Figure 9 shows BUN levels before and after the administration of briplatin (cisplatin) to mice during the administration of PTN or physiological saline.

Figure 10 shows the serum creatinine of the mice in Figure 9.

Best Mode for Implementing the Invention

The present invention is illustrated in detail below with reference to examples, but is not to be construed as being limited thereto.

Example 1: Effect of MK on relieving nephropathy *in vivo*

The 129/Sv knockout mice in which parts of exons 2 and 3 of MK genes were destroyed as illustrated in Figure 1 were used (Biochemistry 7, Volume 68, pp 1239, 4-p-1244, 1996). Figure 2 shows the survival rate of the 129/Sv MK gene-knocked out mice and that of wild mice after the intraperitoneal administration of 14 mg/kg cisplatin (product name, briplatin, Bristol Myers Squibb Company). Figure 3 shows BUN levels 0, 3, and 5 days after the administration of the cisplatin. Cisplatin was selected because it is the fastest, most effective, and most common antitumor agent against solid tumors. Moreover, the side effect of cisplatin is nephropathy such as acute renal failure and MK is expressed only in kidneys in adult mice.

The Student t test revealed that the BUN levels of knockout mice were significantly higher than that of wild mice, as indicated in Figure 3.

Figure 4 shows the frequency of abnormal blood urea nitrogen of mice at zero, three, and five days after the administration of 14 mg/kg of cisplatin; 207 mg of MK-containing sustained release capsules were intraperitoneally implanted in seven mice of the MK administration group , and 207 mg of physiological saline-containing sustained release capsules were intraperitoneally implanted in seven mice of the physiological saline administration group. The frequency of abnormality indicates the rate of occurrence of BUN abnormality when 50 BUN or more is the abnormal level.

Example 2: Effect of MK on relieving hepatopathy

Wild mice with acute hepatopathy caused by carbon tetrachloride were prepared. The effects of administering MK on relieving acute hepatopathy were then monitored. Five mice were employed for each treatment. Once carbon tetrachloride diluted to 10% with food oil "Medium Chain Triglyceride: Panasate 800" (NOF corporation) were administered to the mice, they were fasted. Physiological saline,

0.017 mg of MK or 1.7mg of MK (JP-A No. Hei 9-95454), was administered intraperitoneally after 24 hours and again 8 hours after the first administration. Blood was collected after 16 hours, and serum GOT and GPT were measured (Figures 5 and 6).

The Student t test revealed that the GOT of the physiological saline group did not significantly differ from that of the MK (1.7 mg) administration group as shown in Figures 5 and 6. In contrast, GPT between the groups differed significantly at the 5% risk level. It was thus determined that MK significantly relieved acute hepatopathy caused by carbon tetrachloride.

Example 3: Effect of MK on relieving nephropathy

G401 cells derived from human infantile renal cancer (Wilms tumor) were used considering the side effects of antitumor agent cisplatin on kidneys.

G401 cells derived from human infantile renal cancer (Wilms tumor) were adjusted to 1×10^5 cells/ml or 3×10^5 cells/ml by 10% FBS/DME medium. 2×10^4 cells or 6×10^4 cells were inoculated per well of a 96-well plate (COSTAR: 3596) and incubated at 37℃ overnight under a 5% CO_2 atmosphere.

Subsequently, the cells were incubated in a 0.1% FBS/DME medium containing 2 μg/ml or 10 μg/ml of MK and in the same medium without MK as a control group for six hours. After the second incubation, the cells were incubated in a medium containing 10 μM of cisplatin (product name, briplatin, Bristol Myers Squibb Company) for 2 hours.

The cultured media were washed four times after the incubation, and the cell incubation was continued in a media containing 2 μg/ml or 10 μg/ml of MK the same as above.

Proliferation activity of the live cells was measured with Premix WST-1 Cell Proliferation Assay System (Takara) to evaluate the effect of MK on relieving nephropathy. Proliferation activity was assayed by intracellular division and proliferation of mitochondria reflected in absorbance (450 nm, control: 655 nm) in the same way as in an MTT assay.

Specifically, the cells were treated by cisplatin. Premix WST-1 reagent, up to 1/10 of the medium, was then added to each well on

the second, third, or fourth day after the cispaltin treatment, and the cells were incubated for 4 hours. The absorbance of each well was measured with a Plate Reader (BIO-RAD; Model 3550) (Figures 7 and 8). Figures 7 and 8 indicate that 2 to 10 μg/ml of MK dramatically decreased the number of G401 cell deaths due to cisplatin (antitumor agent) nearly two fold.

Example 4: Effect of PTN on relieving drug-induced nephropathy

ICR mice (male, 8 to 10 week-old) were divided into two groups; one for physiological saline administration and the other for PTN administration (11 mice each). The dosages of PTN and physiological saline were 500 μg/kg for each group (Merenmies, J. and H. Rauvala: J. Biol. Chem. 265: 16721-16724, 1990). Briplatin (Bristol Myers Squibb Company) was used as cisplatin.

PTN or physiological saline was administered intraperitoneally to each mouse of the above two groups for three days continuously. On the fourth day, whole blood was collected from three mice of each group, and serum was prepared to serve as the serum sample before the administration of briplatin.

In the afternoon of the same day (the fourth day), 15 mg/kg of briplatin was intraperitoneally administered to each of the rest of mice. PTN or physiological saline was continuously administered to the rest of mice on each day until the seventh day. On the sixth and eighth days, whole blood was collected from the mice, and the serum was prepared to serve as the serum sample on the second and fourth days after the administration of briplatin.

Blood urea nitrogen (BUN), a representative marker of renal function, (Figure 9) and serum creatinine (Figure 10) of each sample were measured by Iatro-chrom UN (IATRON LABORATORIES, INC.) and Creatinine-test Wako (Wako Pure Chemical Industries, Ltd.), respectively. Figures 9 and 10 show that BUN and creatinine in the physiological saline administration group tended to be higher than that in the PTN administration group. These results indicate that both PTN and MK relieve drug-induced nephropathy.

Industrial Applicability

The present invention demonstrates that the MK family proteins effectively relieve drug-induced nephropathy and hepatopathy. Therefore, pharmaceutical composition of the invention, comprising MK family protein as an effective ingredient, is useful for relieving nephropathy and hepatopathy induced by drugs, especially antitumor agents.

CLAIMS

1. A pharmaceutical composition for treating or preventing drug-induced nephropathy or hepatopathy, comprising an MK family protein as an effective ingredient.

2. The pharmaceutical composition of claim 1, wherein said composition treats or prevents drug-induced nephropathy caused by the administration of an antitumor agent.

3. The pharmaceutical composition of claim 2, wherein said antitumor agent is cisplatin.

4. Use of an MK family protein for preparing a pharmaceutical composition for treating or preventing drug-induced nephropathy or hepatopathy.

5. The use of an MK family protein of claim 4, wherein said drug-induced nephropathy is caused by an antitumor agent.

6. The use of an MK family protein of claim 4, wherein said antitumor agent is cisplatin.

7. A method for relieving drug-induced nephropathy or hepatopathy, which comprises administering an MK family protein.

8. The method for relieving drug-induced nephropathy or hepatopathy of claim 7, wherein said drug-induced nephropathy is caused by the administration of an antitumor agent.

9. The method for relieving drug-induced nephropathy or hepatopathy of claim 7, wherein said antitumor agent is cisplatin.

ABSTRACT

The present invention provides a novel drug for relieving drug-induced nephropathy and acute hepatopahy containing a midkine (MK) family protein such as pleiotrophin (PTN). The MK family proteins can inhibit nephropathy induced by an antitumor agent or acute hepatopathy caused by carbon tetrachloride and thus effectively relieve drug-induced nephropathy or hepatopathy.

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[54] 发明名称 用于药物诱导型肾病和肝病的预防或治疗组合物

[57] 摘要

本发明提供缓解药物诱导型肾病和急性肝炎的一种新药，包括 midkine (MK) 家族的蛋白质如 pleiotrophin(PTN)。这些 MK 家族的蛋白质可抑制抗肿瘤制剂如顺铂诱导的肾病和四氯化碳引起的急性肝炎，从而有效缓解药物诱导型肾病或肝炎。

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知识产权出版社出版

1. 一种用于治疗或预防药物诱导型肾病或肝病的药物组合物，其中所述药物组合物含MK或PTN作为有效成分，所述诱导肾病的药物是顺铂或另一种与顺铂以相同机制发挥作用的抗肿瘤制剂，所述诱导肝病的药物是四氯化碳。

2. 权利要求1的药物组合物，其中所述诱导肾病的药物是顺铂。

3. 应用MK或PTN制备治疗或预防药物诱导型肾病或肝病的药物的用途，其中所述诱导肾病的药物是顺铂或另一种与顺铂以相同机制发挥作用的抗肿瘤制剂，所述诱导肝病的药物是四氯化碳。

4. 权利要求3的用途，其中所述诱导肾病的药物为顺铂。

用于药物诱导型肾病和肝病的预防或治疗组合物

技术领域

本发明涉及用于缓解药物诱导型肾病和肝病的药物.

背景技术

治疗和/或诊断性药物的应用逐年增大，所用药物多种多样.这些药物可为我们带来显著的好处，但也可实质性导致损害效应，尤其是对肾脏，这是因为它们的如下所述的具体作用.

肾脏在总体重中占不到 1%.从生理学的角度看，心脏输出血流总量的25%进入肾脏，每天有 150 升原尿(为总血浆量的 50 倍之多)经肾小球过滤;再经结构和功能多相性不相同的肾细管再吸收，分泌和代谢便产生最终的尿.这样一来，血中药物及其代谢物不断地循环，而这些物质在肾脏浓缩和代谢.因此，各种高度浓缩的代谢物包括未代谢的药物分散在肾脏中.肾脏可能经常性地高强度地接触药物.四类药物可能诱导肾病:抗微生物试剂，非类固醇剂，造影剂，和抗肿瘤制剂.

肝脏也易受药物损伤.药物诱导型肝病根据其发病机制分为由药物或其中间代谢产物直接攻击肝脏引起的中毒性肝病，以及由变态反应，涉及 T 细胞的IV型迟发型变态反应引起的变态反应性肝病.药物诱导型肝炎最常见的致病因素有抗生素，其次有用于中枢神经系统的药物，用于循环器官的药物，抗肿瘤制剂、激素、诊断试剂，等等.

已尝试用 γ－球蛋白，细胞色素 C，腺嘌呤，SH 化合物，B 族维生素等缓解药物诱导型疾病，但它们均非足够有效.很重要的是临床治愈药物诱导型疾病(副作用)，因为治疗的中断和病人的生活质量(Q.O.L)的重要性.

发明详述

本发明的一个目标是能有效缓解或抑制由各种药物，尤其是抗肿瘤制剂诱导的疾病的药物.

发明人将注意力集中于以下事实: midkine(MK)家族的蛋白质如midkine(MK)和 pleiotrophin(PTN)是一类具有多种功能的生长和分化因子.其

功能有: 1)延伸轴突, 2)活化纤溶系统, 3)强烈表达于人体癌症区, 及 4)创伤的治愈. 关于这类蛋白质进行了很多研究, 目的是寻找新的药效.

Midkine 是作为一种基因产物被发现的, 该基因在小鼠胚胎肿瘤细胞的分化早期受视黄酸诱导而表达(Kadomatsu, K.等, 生物化学与生物物理学研究通讯, 151:1312-1318, 1988). Pleiotrophin 发现于一只新生大鼠的脑内, 是有轴突延伸能力的肝素结合蛋白(Rauvala, H., EMBO J., 8:2933-2941, 1989). Midkine 和 Pleiotrophin 构成了一类新的肝素结合蛋白类的生长分化因子. 它们之间有 45%同源性并总称 MK 家族(Muramatsu, T.,国际发育生物学杂志, 37:183-188, 1993; Muramatsu, T., 生长与分化进展, 36(1):1-8, 1994). Midkine 和 Pleiotrophin 分别在发育进程中有其特殊的表达模式, 并且预计涉及分化过程中重要的生理性活化作用.

本发明人发现, MK 体外抑制抗肿瘤制剂所致细胞死亡, 而 MK 基因缓解抗肿瘤制剂诱导的疾病, 而这些发现均源于一个实验结果, 实验中对 MK 基因功能受破坏的基因敲除小鼠施用抗肿瘤制剂. 发明人还发现对野生型小鼠施用 MK 或 PTN 可缓解抗肿瘤制剂所致疾病, 从而完成本发明. 本发明包括权利要求书中所述的每项发明.

在本发明中, 基因敲除小鼠提供机会便于调查 MK 基因在活体内如何对抗药物所致疾病, 还便于分析每只基因敲除小鼠如何对被迫接受不同水平的 MK 给药进行应答. MK 的详细功能和机制目前尚不清楚. 如果 MK 的功能是细胞因子或生长因子功能级联启动蛋白, 则大概只需极少量的 MK, 而且基因敲除小鼠的应用将变得更为重要. 最近发现一种以高亲和力特异性结合 MK 的细胞表面受体(分子量 250+kDa). 其特点暗示肿瘤细胞增殖中由 MK 所刺激的自分泌可由该受体介导, 并可活化 JAK/STAT 途径(Edward, A.R.等, 生物化学杂志, 273:3654-3660, 1998).

为弄清 MK 与个体发育的关系, 制备了纯合型 MK 基因敲除小鼠, 其中外显子 2 和外显子 3 部分被破坏, 如图 1 所示(生物化学 7, Heisei 8:卷 68, 1239 页, 4-p-1244). 这些基因敲除小鼠在胚胎期并未死亡, 体重明显低于杂合型或野生型(生物化学 7, 卷 68, 1239 页, 4-p-1244, 1996).

抗肿瘤试剂被施予这些基因敲除小鼠(简称基因敲除小鼠)和野生型小鼠. 给药后比较每只小鼠的存活率, 血尿素氮(BUN)水平, 肌酸酐水平作为这些疾病指标, 以便监控 MK 基因活体内缓解抗肿瘤制剂所致疾病的能力.

BUN和肌酸酐水平可应用作为肾功能障碍的指标，因为由于肾排泄能力降低将使尿素积累于血液.

在本发明中，对基因敲除小鼠和野生型小鼠施用顺铂，再比较 BUN 水平和存活率. 基因敲除小鼠的 BUN 水平显著高于野生型小鼠的. 基因敲除小鼠的存活率也明显区别于野生型小鼠的. 基因敲除小鼠的死亡率截止到给药后第 7 天上升. 被迫接受 MK 的基因敲除小鼠中异常 BUN 水平的发生率截止到给药后第 3 天明显低于给予生理盐水的小鼠组. 用人婴儿肾癌细胞系进行一项体外实验证实 MK 抑制由顺铂引起的肾细胞障碍的效应. 应用野生型小鼠的一项实验揭示 MK 缓解了由四氯化碳引起的急性肝病，而 PTN 和 MK 均能有效抑制顺铂引起的肾病.

这些结果说明本发明的MK家族的蛋白质可有效缓解或抑制药物诱导型肾病和肝病.

用作本发明之药物组合物的有效成分的 MK 蛋白(简称 MK)述于以下参考文献(人 MK 基因，未审查已公布的日本专利申请(JP-A)号 Hei 5-91880；人 MK 基因和蛋白的序列，JP-A 号 Hei 6-217778；MK 蛋白，JP-A 号 Hei 5-229957；Muramatsu, T.，生长与分化进展，36(1), 1-8, 1994). 用作本发明之药物组合物的有效成分的PTN蛋白述于以下文献(Muramatsu, T.，生长与分化进展，36(1), 1-8, 1994；Andreas, K 等，肿瘤发生的主要综述，6(2):151-177, 1995). 属于 MK 家族并用作本发明之药物组合物的有效成分的蛋白质包括来自人，小鼠或其它动物的天然蛋白，或经化学合成或遗传工程制成的人工蛋白. 本发明的 MK 家族蛋白质还包括不引起任何上述生物学活性改变并与天然蛋白有氨基酸的数量或序列差异的蛋白，本发明具体包括这样的蛋白，它对应于天然蛋白，但其中天然蛋白的氨基酸序列部分缺失或被其它氨基酸取代，或插入或增加了不同长度的其它氨基酸或多肽. 被取代或插入的氨基酸不限于天然型的.

可用大肠杆菌表达系统(Studier, F.W.& Moffatt, B.A., 分子生物学杂志 189:113-130, 1989; Studier, F.W.等，酶学方法 185:60-89, 1990)或杆状病毒表达系统(O'Reilly, D.R.等，杆状病毒表达载体实验室手册，牛津大学出版社，1992，Ausubel, F.M.等编辑，分子生物学最新实验程序，16.11 单元，Wiley Intescience, 1994)经遗传工程技术获得作为本发明有效成分的 MK 家族蛋白. 发明人用甲醇依赖型巴斯德毕赤氏酵母(Pichia Pastoris)的表达系统获得

MK 蛋白(参见 JP-A 平 9-95454).

本发明的药物组合物包含有效量的 MK 家族的蛋白，以防或治药物引起的肾病或肝病. 本发明的有效成分可制成所需剂型，只需将之混合于常用的可药用载体，赋形剂，稀释剂，防腐剂，稳定剂，缓冲液等等.

本发明的药学组合物可口服或非经肠给药. 口服的剂型包括片剂，粒剂和胶囊. 非经肠给药的剂型包括注射液，栓剂或透皮剂，给药途径有静脉内，皮下，肌肉内或腹膜内.

生理活性肽如 MK 或 PTN 若口服一般在消化道内被蛋白酶迅速降解. 为在体内稳定 MK 或 PTN，可结合水溶性大分子(如聚乙二醇(PEG)或聚乙烯吡咯烷酮(PVP))而制成杂交 MK 或杂交 PTN. IL-6， TNF-α 等的杂交构建已有人尝试，经选择最适杂交条件可增强功能(Tsutsumi, Y.等，英国癌症杂志 74:1090-1095; Tsutsumi, Y.等， Thoromb Haemostasis, 77:168-173, 1997; Tsutsumi, Y.等， J Control Release, 33:447-451, 1995).

作为本发明之药物组合物有效成分的MK家族的蛋白质根据用于防或治药物所致肾病或肝病时致病药物的剂量，肾病或肝病的严重程度，病人的年龄、性别和体重而有变化. 本发明的蛋白每天可给予一次或多次，剂量为每 kg 体重 1μg-100mg.

图的简述

图 1 显示基因敲除小鼠的突变染色体，其中 129/sv 小鼠的 MK 基因的外显子 2 和 3 的部分被破坏.

图 2 显示 129/Sv MK 基因敲除小鼠及野生鼠在给予顺铂之后的存活率.

图 3 显示 129/Sv MK 基因敲除小鼠及野生鼠在给药顺铂的当天，第 3 天和第 5 天的血尿素氮水平.

图 4 显示 129/Sv MK 基因敲除小鼠在给予了 14mg/kg 顺铂并腹膜内埋植了已吸收有 MK 或生理盐水的缓释胶囊后，在给药的当天，第 3 和第 5 天异常血尿素氮的发生率.

图 5 显示有四氯化碳所致急性肝病的小鼠在施用了不同浓度的 MK 或生理盐水后的血清 GOT(谷草转氨酶).

图 6 显示相同于图 5 的小鼠的血清 GTP(谷丙转氨酶).

图 7 显示 2×10^4 细胞/孔的 G401 细胞在单独的培养基，加有 2μg/ml MK 的培养基，或加有 10μg/ml MK 的培养基，及培养过程中用 briplatin(顺铂)处

理的细胞存活率。

图 8 显示 G401 细胞(6×10^4 细胞/孔)经图 7 中同样的处理后的细胞存活率。

图 9 显示小鼠在施用 PTN 或生理盐水期间，给药 briplatin(顺铂)之前和之后的 BUN 水平。

图 10 显示图 9 小鼠的血清肌酸酐水平。

实现本发明的最佳方式

本发明参照以下实施例详细举例说明，但不限于此。

实施例 1: MK 体内缓解肾病的效应

使用 129/Sv 基因敲除小鼠，其 MK 基因的外显子 2 和 3 的部分被破坏，如图 1 所示(生物化学 7，卷 68，1239 页，4-p-1244，1996，Nakamura, E. 等，Genes to Cells 3, 811-822, 1998)。图 2 显示了 129/Sv MK 基因敲除小鼠及野生型小鼠腹膜内接受 14mg/kg 顺铂(产品名，briplatin，Bristol Myers Squibb 公司)后的存活率。图 3 显示给予顺铂后当天，第 3 天和第 5 天的 BUN 水平。选择顺铂是因为它是最快，最有效，且最常见的抗实体瘤抗肿瘤制剂。而且，顺铂的副作用是肾病如急性肾衰而 MK 只表达于成年小鼠的肾脏中。

Student t 检验揭示基因敲除小鼠的 BUN 水平显著高于野生型小鼠，如图 3 所示。

图 4 显示给予 14mg/kg 顺铂的当天，第 3 天和第 5 天小鼠的异常血尿素氮发生率；207mg 含 MK 的缓释胶囊被腹膜内植入 MK 给药组的七只小鼠，207mg 含生理盐水的缓释胶囊被腹膜内植入生理盐水给药组的七只小鼠。异常率说明当 50 BUN 或更多作为异常水平时的 BUN 异常发生率。

实施例 2: MK 缓解肝病的效应

制备由四氯化碳引起急性肝病的野生型鼠。然后监测施用 MK 对缓解急性肝病的效应。每个处理用 5 只小鼠。一旦用食物油“中等链长的甘油三酯: Panasate 800” (NOF 公司)稀释至 10%的四氯化碳对小鼠施用，小鼠被禁食。生理盐水，0.017mg MK 或 1.7mg MK(JP-A 号 Hei 9-95454)分别在第一次给药的 24 小时后和 32 小时后腹膜内施用。16 小时后收集血液，测量血清 GOT(谷草转氨酶)和 GPT(谷丙转氨酶)(图 5 和 6)。

Student t 检验揭示生理盐水组的 GOT 与 MK(1.7mg)给药组的 GOT 并无显著性差异，如图 5 和 6 所示。相比之下，组间 GPT 有 5%风险水平的显著

性差异. 因此可确定MK显著缓解了四氯化碳所引发的急性肝病.

实施例3： MK缓解肾病的效应

人婴儿肾癌(Wilms 肿瘤)源的 G401 细胞被用于检验抗肿瘤制剂顺铂对肾脏的副作用.

人婴儿肾癌(Wilms 肿瘤)源的G401细胞用10% FBS/DME培养基调成1 × 10^5细胞/ml或3 × 10^5细胞/ml. 在96孔板(COSTAR:3596)上每孔接种2 × 10^4细胞或6 × 10^4细胞, 37 ℃ 5%CO_2气氛中温育过夜.

然后, 将细胞温育在含或不含(对照)2μg/ml 或 10μg/ml MK 的 0.1% FBS/DME培养基中6小时. 第二次接种后, 将细胞于含10μM顺铂(商品名, briplatin, Bristol Myers Squibb公司)的培养基中温育2小时.

温育后将培养基洗四次, 再继续将细胞温育于含2μg/ml或10μg/ml MK的培养基(同上)中.

同 Premix WST-1 细胞增殖试验系统(Takara)测量活细胞的增殖活性以评估 MK 缓解肾病的效应. 通过反映为吸收值(450nm , 对照: 650nm)的线粒体胞内分裂和增殖分析增殖活性, 就象MTT试验的方式一样.

具体地细胞用顺铂处理. 处理后第二, 三, 四天每孔再加入多达培养液的1/10的Premix WST-1试剂, 然后温育细胞4小时. 用Plate Reader(BIO-RAD; 3550型)测量每孔的吸收值(图7和8). 图7和8说明2-10μg/ml MK使顺铂(抗肿瘤制剂)引起的G401细胞死亡数显著降低了近2倍.

实施例4： PTN缓解药物诱导型肾病的效应

ICR小鼠(雄性, 8-10周龄)分成两组; 一为生理盐水给药组, 另一为PTN给药组(每组11只小鼠). PTN和生理盐水的剂量为每组500μg /kg(Merenmies, J.和 H. Rauvala: 生理化学杂志, 265:16721-16724, 1990). 用Briplatin(Bristol Myers Squibb公司)作为顺铂.

连续三天对上述两组的每只小鼠腹膜内施用PTN或生理盐水. 第4天, 每组收集3只小鼠的全血, 制备血清作为briplatin用药前的血清样本.

同一天(第4天)下午, 对剩下的每只小鼠腹膜内给予15mg/kg briplatin. 连续每天对所剩每只小鼠给予PTN或生理盐水直至第七天. 在第六和第八天, 收集小鼠全血, 制成血清, 作为briplatin给药后第2和第4天的血清样本.

血尿素氮(BUN)是肾功能的代表性标志物. 用 Iatro-chrom UN(IATRON

LABORATORIES, 公司)和肌酸酐－检验 Wako(Wako Pure 化学工业公司)分别测量每份样本的 BUN(图 9)和血清肌酸酐(图 10). 图 9 和 10 显示生理盐水给药组的 BUN 和肌酸酐倾向于高过 PTN 给药组的. 这些结果说明 PTN 和 MK 均能缓解药物诱导型肾病.

工业化适用性

本发明证实 MK 家族的蛋白质可有效缓解药物诱导型肾病和肝病. 因此, 包含 MK 家族的蛋白质作为有效成分的本发明之药物组合物可用于缓解由药物, 尤其是抗肿瘤制剂诱导的肾病和肝病.

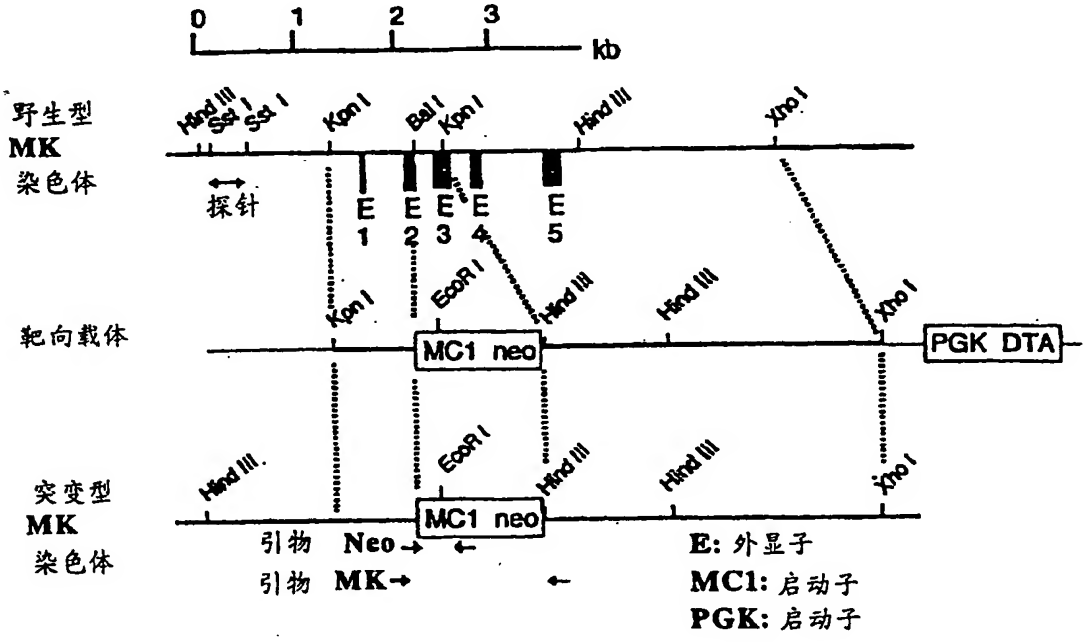
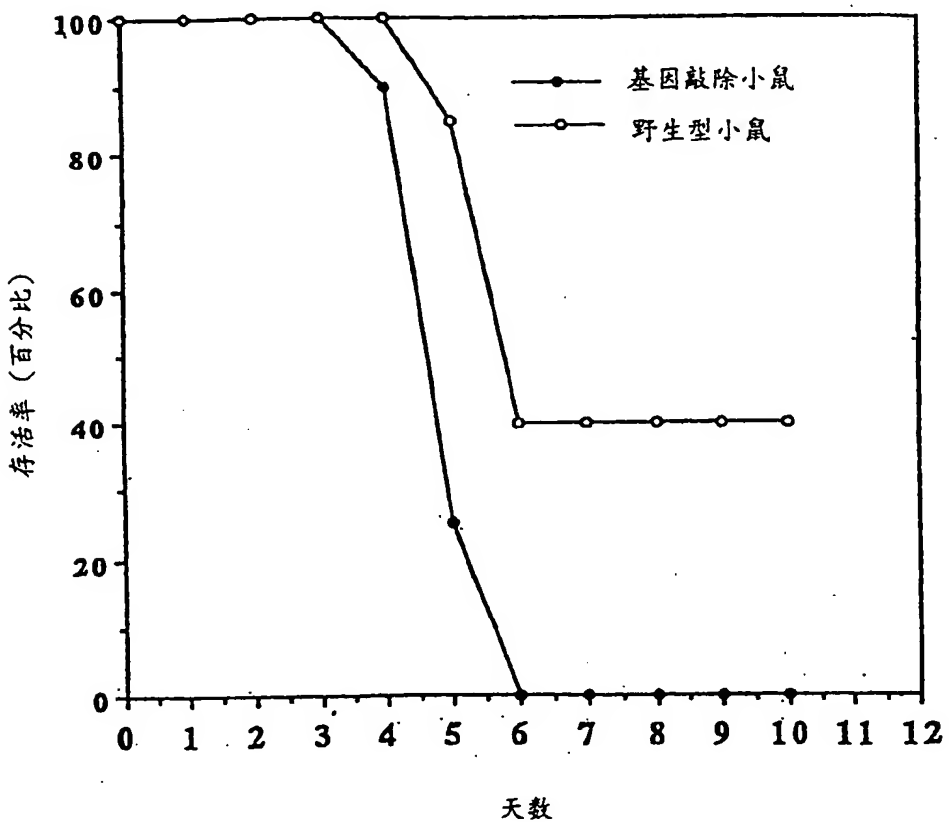

0
1
2
3
kb
野生型
MK
染色体
Hind III
Sst I
Sst I
Kpn I
Bal I
Kpn I
Hind III
Xho I
探针
E 1
E 2
E 3
E 4
E 5
靶向载体
Kpn I
EcoR I
Hind III
Hind III
Xho I
MC1 neo
PGK DTA
突变型
MK
染色体
Hind III
EcoR I
Hind III
Hind III
Xho I
MC1 neo
引物 Neo→
←
引物 MK→
←
E: 外显子
MC1: 启动子
PGK: 启动子

图 1


100
80
60
40
20
0
存活率（百分比）
0 1 2 3 4 5 6 7 8 9 10 11 12
天数
基因敲除小鼠
野生型小鼠

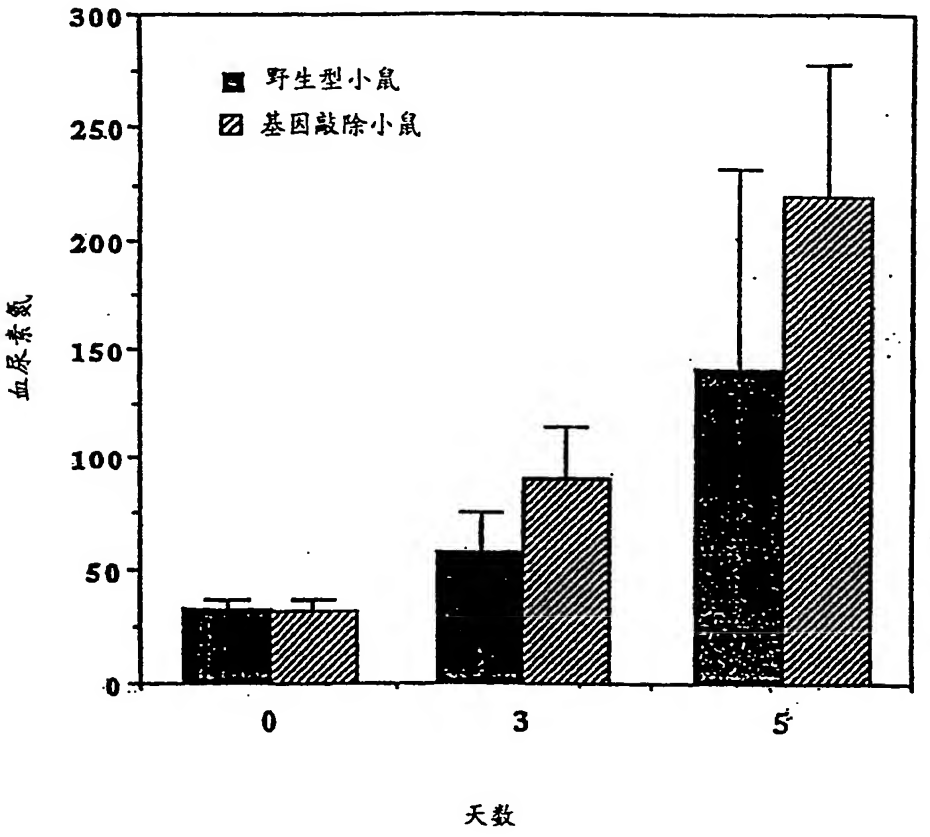


图 3

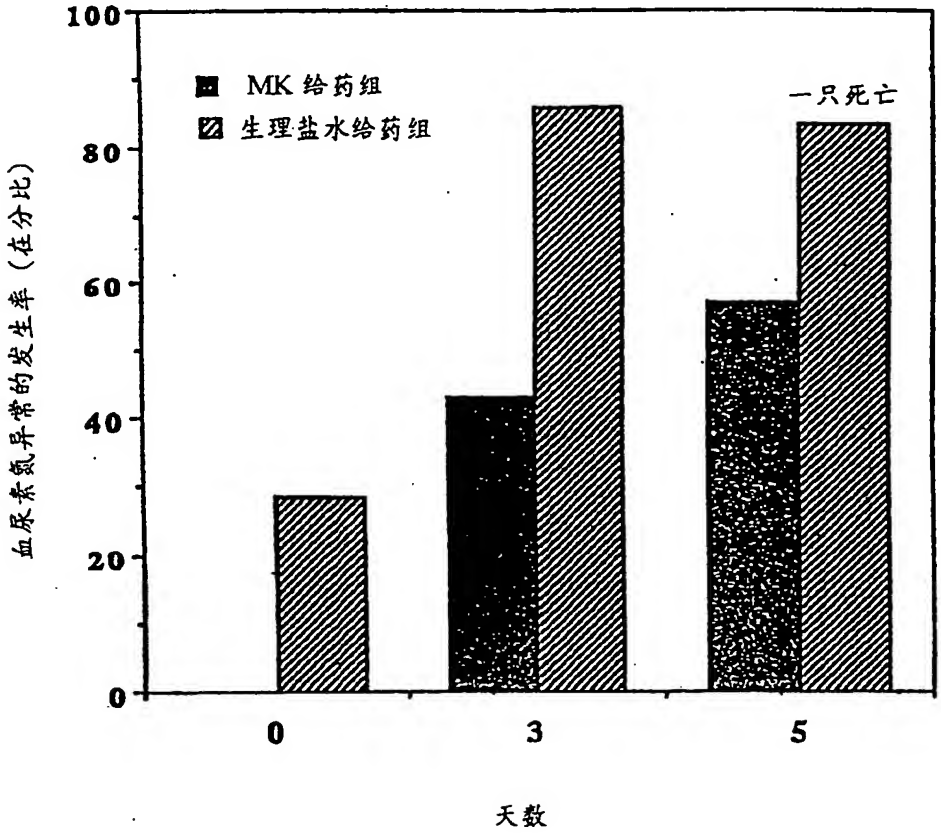


图 4

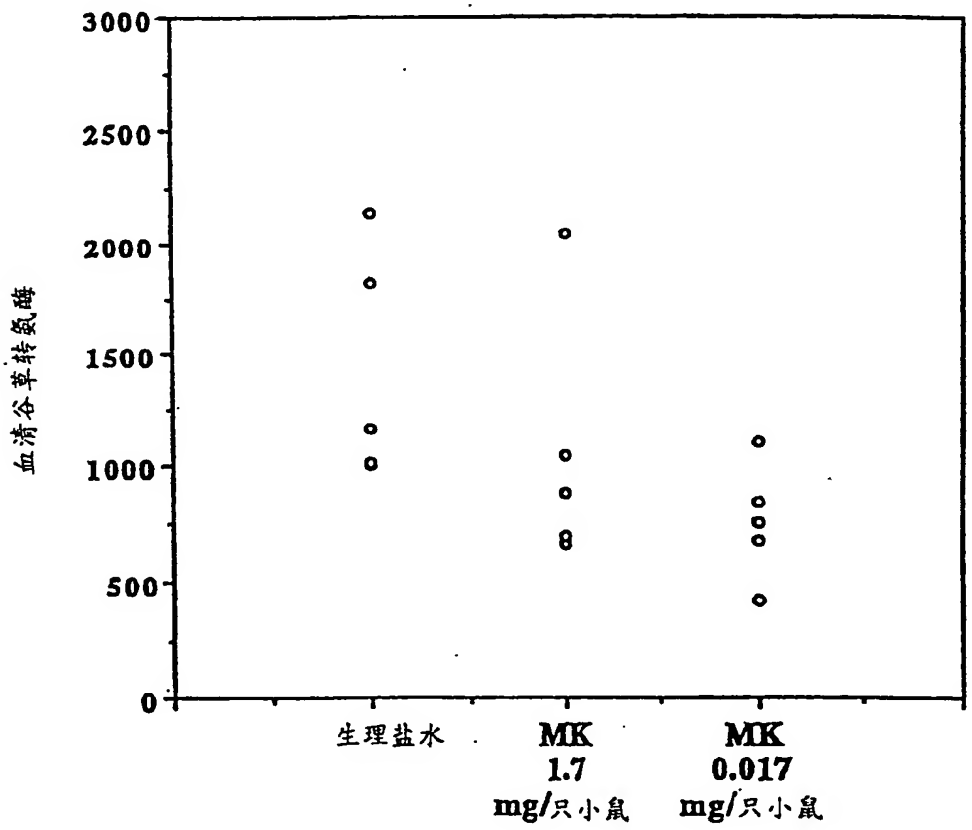

3000
2500
2000
1500
1000
500
0
血清谷草转氨酶
生理盐水
MK
1.7
mg/只小鼠
MK
0.017
mg/只小鼠

图 5

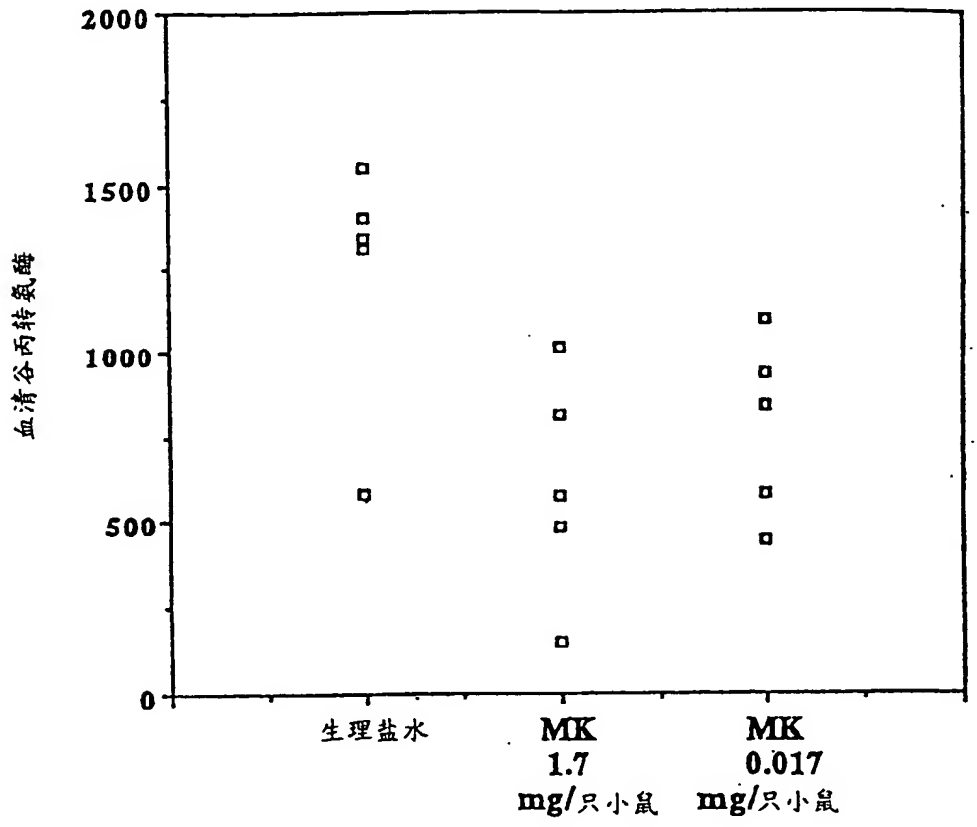


图 6

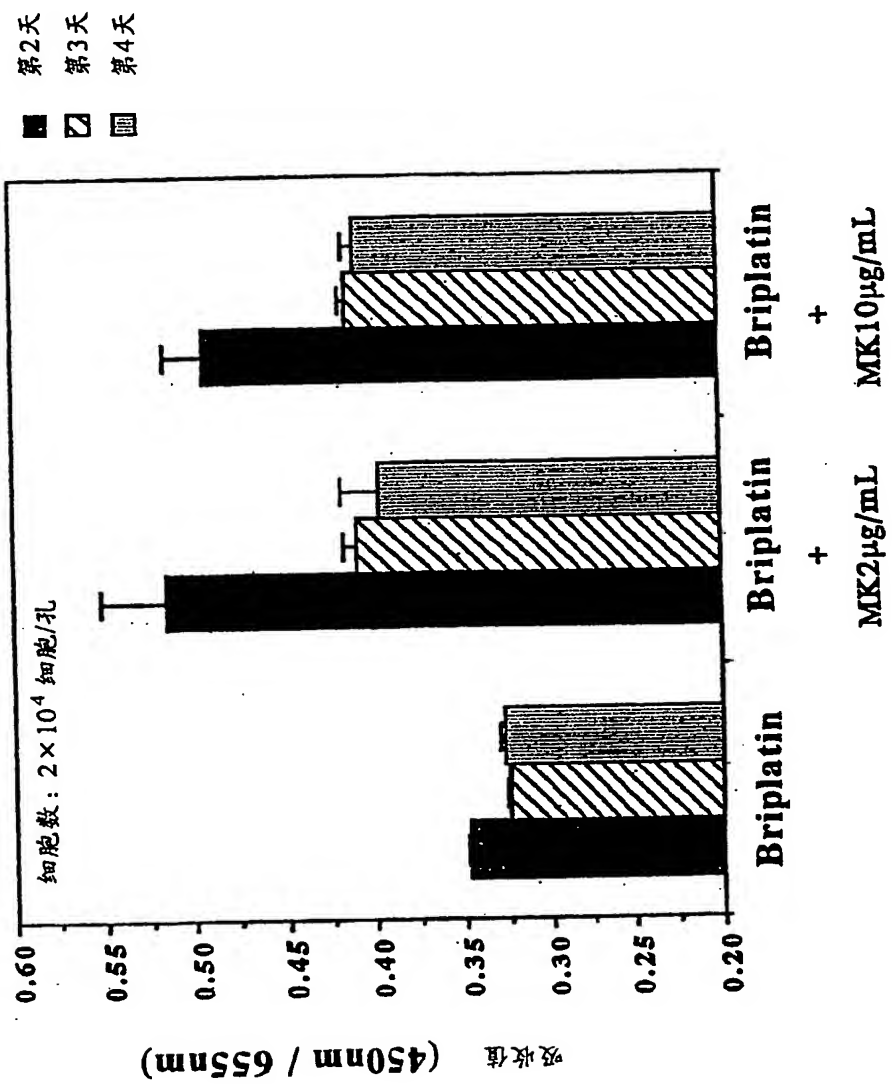

第2天
第3天
第4天
细胞数：2×10^4 细胞/孔
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
吸光值 (450nm / 655nm)
Briplatin
Briplatin + MK2μg/mL
Briplatin + MK10μg/mL

图 7

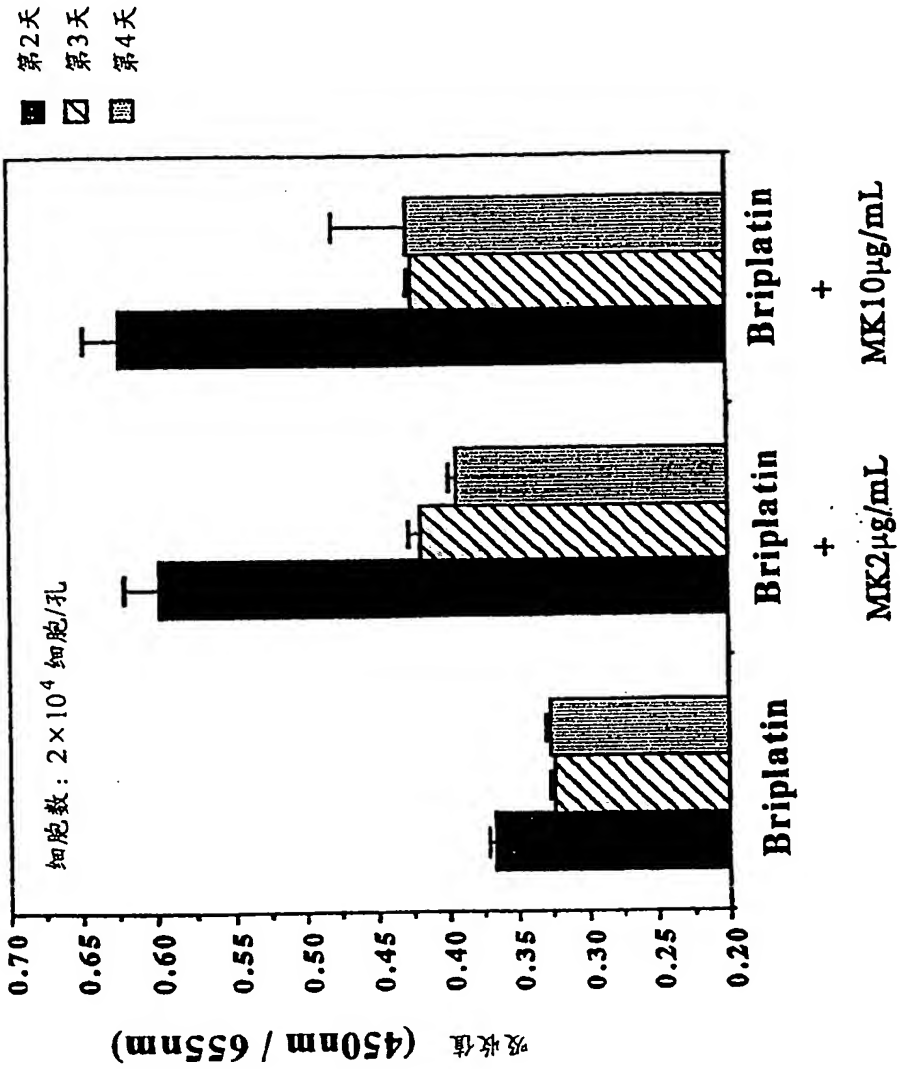

第2天
第3天
第4天
细胞数：2×10^4 细胞/孔
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
吸光值 (450nm / 655nm)
Briplatin
Briplatin + MK2μg/mL
Briplatin + MK10μg/mL

图 8

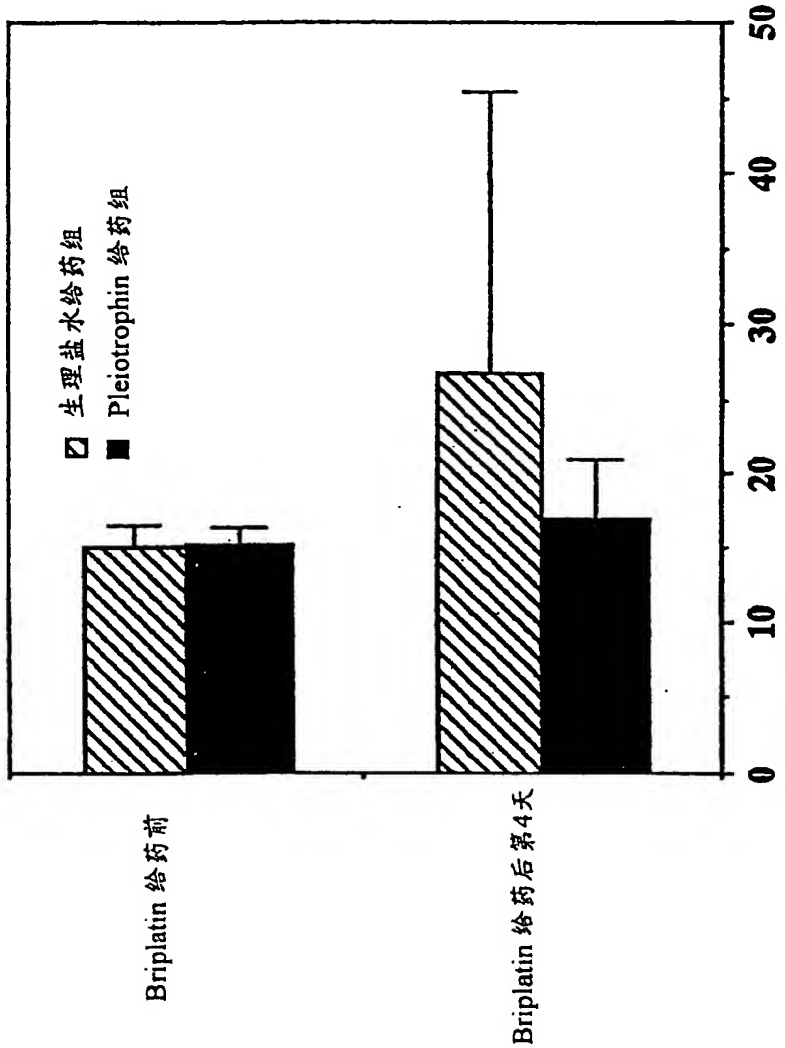

生理盐水给药组
Pleiotrophin 给药组
0
10
20
30
40
50
血尿素氮（mg/dL）
Briplatin 给药前
Briplatin 给药后第4天

图 9

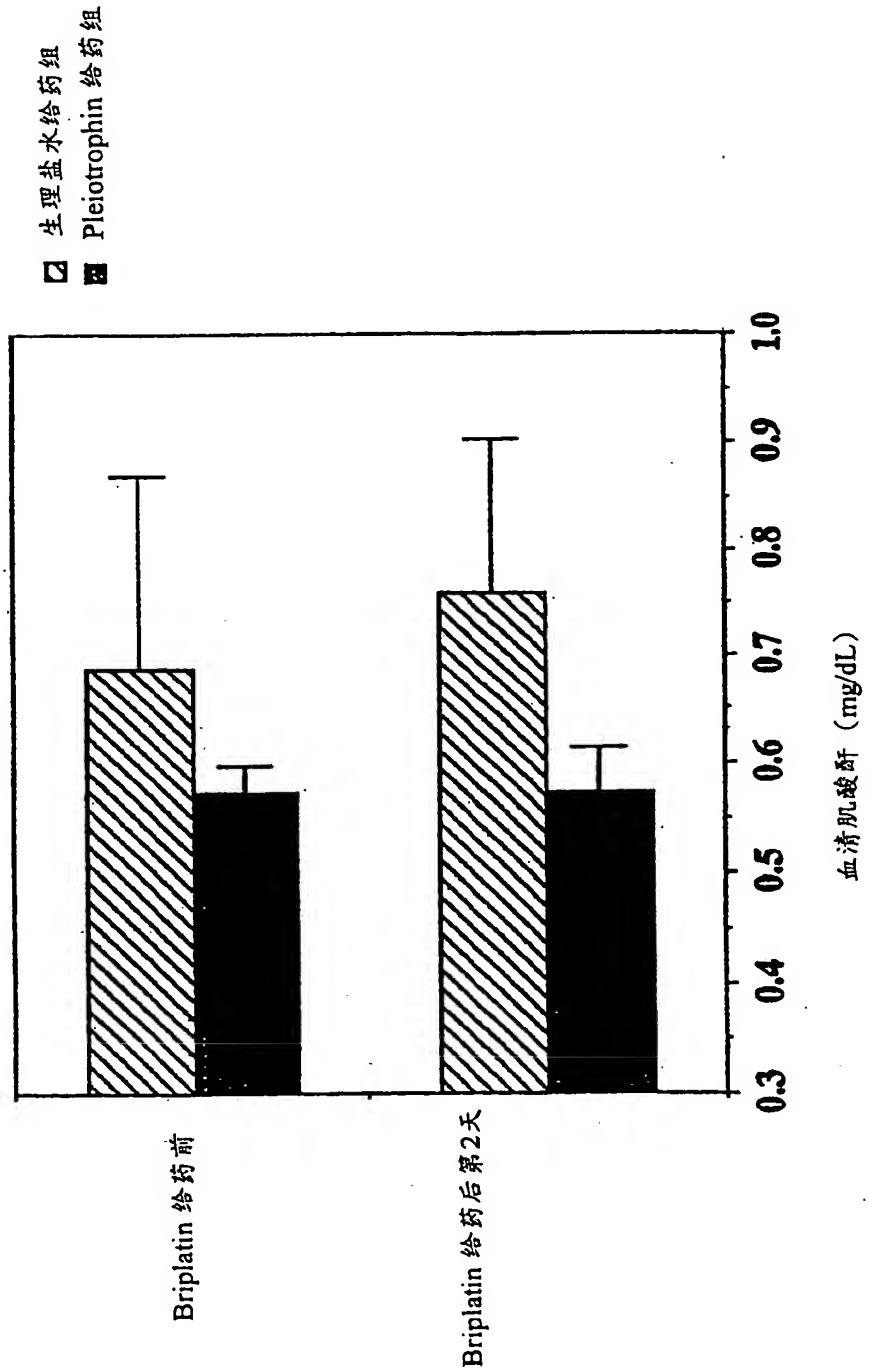

生理盐水给药组
Pleiotrophin 给药组
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
血清肌酸酐（mg/dL）
Briplatin 给药前
Briplatin 给药后第2天

图 10